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TUNGSTEN-INDUCED INACTIVATION OF MOLYBDOENZYMES IN *ANABAENA*

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Summary

The effect of tungsten on growth and activity of two molybdoenzymes has been studied in a nitrogen-fixing heterocystous cyanobacterium, *Anabaena*. Sodium tungstate inhibited growth and inactivated nitrogenase and nitrate reductase. The activity of both enzymes was restored by the addition of molybdenum. Tungstate treatment caused increase in heterocyst frequency both in NO_3^- medium and in medium free of combined nitrogen. These results suggest that tungstate treatment inactivates the molybdoenzymes in this cyanobacterium.

Cyanobacteria reduce nitrate and atmospheric nitrogen via convergent metabolic pathways to ammonia by nitrate reductase and nitrogenase, respectively. The enzyme nitrogenase consists of two components viz., component I, which contains Mo—Fe, and acid-labile sulfur, and component II, which contains Fe and acid-labile sulfur [1]. The fact that the end product of both nitrogen and nitrate reduction pathways is ammonia, and that molybdenum is the iso-metal prosthetic group of both the enzymes has led to the suggestion that these enzymes may share a common ammonium-repressible, molybdenum-activating cofactor of low molecular weight. However, molybdenum cofactors from different molybdoenzymes have recently been isolated and purified and it has been shown that nitrogenase and nitrate reductase do not share a common molybdenum cofactor at least in *Klebsiella pneumoniae* [2]. Since the chemical properties of tungsten are quite similar to those of molybdenum (Group VIB elements), tungsten has been frequently employed in studying the formation, kinetics, activity and configuration of various molybdoenzymes [3]. In nitrogen-fixing bacterium *Azotobacter*, addition of tungsten during growth resulted in formation of

defective component I in which tungsten appeared to be substituted for molybdenum [4,5]. Subsequently, it has been reported that tungsten can be replaced and component I can be activated by the addition of Fe-Mo co-factor in vitro [6]. However, very little work has been done on tungsten-induced alteration in enzyme synthesis and/or activity in cyanobacteria. Recently in a nonheterocystous cyanobacterium *Plectonema* sp., tungsten-induced inhibition of nitrogenase activity and formation of defective component I has been shown [7]. In contrast, Tyagi [8] has reported a stimulatory effect of sodium tungstate on growth of *Anabaena doliolum*. We report here tungsten-induced inhibition of growth and inactivation of molybdoenzymes in a nitrogen-fixing heterocystous *Anabaena* sp.

The test organism *Anabaena* sp., a local isolate, was grown axenically in Allen and Arnon's [9] medium either free of combined nitrogen or supplemented with 10 mM NO_3^-. The alga forms heterocysts in a regular spaced pattern (approx. 5.5%) within 24 h after transfer in heterocyst-induction medium. Growth was estimated by extracting chlorophyll after the method of Mackinney [10]. Heterocyst frequency was estimated by counting heterocysts in at least 10—12 filaments. Nitrogenase activity was measured by acetylene-ethylene assay [11]. Assay was performed in calibrated, triplicate serum bottles of about 7.5 ml capacity. The partial pressure of acetylene was kept at 0.1 atm, and 2-ml cell suspensions were routinely injected in each bottle. Reactions were run for 30 min at 28°C and 3000 lux light intensity. Reactions were terminated by injecting 0.5 ml of 15% (w/v) trichloroacetic acid. Ethylene produced in the reaction vessel was analyzed in a CIS Gas Chromatograph (Baroda, India) fitted with a Porapak R column and a hydrogen flame ionization detector. Nitrate reductase activities (in vivo) were estimated by the method of Camm and Stein [12] and results are based on total nitrite formation.

Table I shows that addition of tungsten in nitrogen-free and NO_3^- media causes marked decrease in final growth yield in contrast to that in molybdenum supplemented control medium. The growth remains unaffected in NO_2^-

TABLE I

EFFECT OF SODIUM TUNGSTATE (Na_2WO_4) IN PRESENCE OF DIFFERENT NITROGEN SOURCES ON PER CENT GROWTH YIELD AND HETEROCYST FREQUENCY OF *ANABAENA* SP

Growth and heterocyst frequency were recorded after 7 and 3 days of inoculation, respectively.

Media	Growth yield (%)	Heterocyst frequency (%)
Control (nitrogen-free)	100	5.5
Nitrogen-free + 100 μg/ml Na_2WO_4	16	14.6
10 mM NO_3^-	100	—
10 mM NO_3^- + 100 μg/ml Na_2WO_4	21	14.0
2 mM NH_4Cl	100	—
2 mM NH_4Cl + 100 μg/ml Na_2WO_4	100	—
2 mM NO_2^-	100	—
2 mM NO_2^- + 100 μg/ml Na_2WO_4	100	—
Nitrogen-free + 100 μg/ml Na_2MoO_4 + 100 μg/ml Na_2WO_4	94	6.2
10 mM NO_3^- + 100 μg/ml Na_2MoO_4 + 100 μg/ml Na_2WO_4	96	—

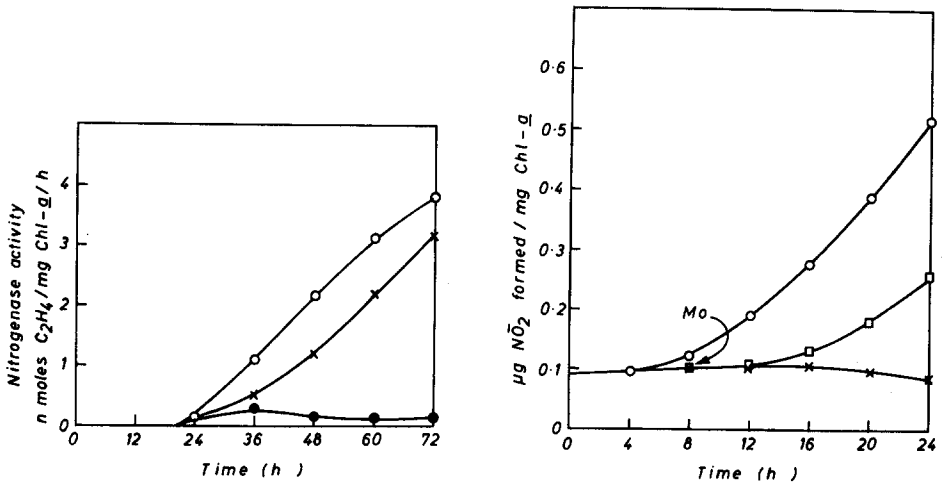

Fig. 1. Effect of Na_2WO_4 on nitrogenase activity of *Anabaena* sp. Culture (NO_3^- grown) was transferred to combined nitrogen-free media with supplementation of Na_2WO_4 and activity was measured at different time intervals as described in text. ○—○, unsupplemented; x—x, Na_2WO_4 plus Na_2MoO_4 (100 μg/ml each, added at 0 h), and ●—●, Na_2WO_4, 100 μg/ml alone.

Fig. 2. Effect of Na_2WO_4 on nitrate reductase activity in *Anabaena*. Culture was grown in NO_3^- media with or without Na_2WO_4. In one set, Na_2MoO_4 was added after 8 h of Na_2WO_4 treatment. NO_2^- formed was estimated at different time intervals. ○—○, NO_3^- control; □—□, NO_3^- + 100 μg/ml Na_2WO_4 and Na_2MoO_4 (100 μg/ml) added after 8 h, and x—x, NO_3^- + 100 μg/ml Na_2WO_4.

and NH_4^+ media containing tungsten. The addition of equal concentrations of molybdenum and tungsten antagonises the growth inhibitory effect of tungsten alone. The heterocyst frequency is greatly increased in nitrogen-free and NO_3^- containing media supplemented with tungsten. Unlike NO_3^- control, in NO_3^- plus tungsten, the frequency increases up to 14%. But the differentiation of heterocysts remains repressed with tungsten treatment in NO_2^- and NH_4^+ media.

Nitrogenase activity (Fig. 1) appeared at about 21 h after transfer of the organism into medium free of combined nitrogen. Maximum activity was observed at 72 h. In tungsten-supplemented medium, minor detectable nitrogenase activity developed upto 36 h followed by a steep decrease, being finally lost at 48 h. In tungsten plus molybdenum containing culture there was parallel development of the enzyme but the activity was lesser than in unsupplemented control as is evident from Fig. 1. There was no appearance of detectable nitrogenase activity following tungsten treatment in NO_3^- media upto 72 h. The nitrate reductase activity (Fig. 2) on the basis of total NO_2^- formation showed an induction period of 6 h and thereafter NO_2^- formation gradually increased upto 24 h in NO_3^- supplemented control sample. But in tungsten-containing medium, no NO_2^- seemed to be formed except a slight increase in its concentration over its basal concentration that occurred within about 8 h of incubation. The addition of molybdenum (100 μg/ml) after 8 h in tungsten-fed culture restored NO_2^- formation after a short lag of 4 h. The heterocyst differentiation was also blocked after the addition of molybdenum.

Our results show that tungsten is inhibitory to growth only under nitrogen-fixing and nitrate-reducing conditions. The enzymes operative in NO_2^- and NH_4^+ metabolism do not seem to be susceptible to tungsten-induced inhibition. The reversal of tungsten-induced growth inhibition by molybdenum indicates that tungsten competitively blocks the utilization of molybdenum. It is known in a variety of organisms that tungsten replaces molybdenum of nitrate reductase and component I of nitrogenase resulting in the formation of defective tungsto-enzymes [3,4,5]. The substitution of tungsten inactivates both the enzymes. In the light of these reports [3,4,5] it may reasonably be concluded that during the growth of *Anabaena* sp. on molecular nitrogen or on nitrate, the respective enzymes nitrogenase and nitrate reductase most probably incorporate tungsten in place of molybdenum. The incorporation of tungsten leads to the formation of defective tungsto-enzymes having no reducing activity for their respective substrates. This is evident from the data of nitrogenase and nitrate reductase activity where there is loss of enzyme activity with tungsten treatment. Moreover, the restoration of activity of both enzymes following molybdenum addition supports the inactivation process of molybdoenzymes with tungsten treatment. However, this is only a reasonable explanation and not a definite conclusion.

Our data on the effect of tungsten treatment on heterocyst differentiation and frequency are in accord with the inactivation of molybdoenzymes hypothesis as heterocyst differentiation remains repressed in media supplemented with NO_2^- and NH_4^+ because the primary enzymes for their assimilation are not molybdoenzymes. The increase in heterocyst frequency in tungsten-treated material without nitrogenase activity may be due to inhibition of nitrogen fixation as a result of the formation of defective tungsto-nitrogenase. This results in nitrogen starvation and induces more vegetative cells to differentiate into heterocysts. Similarly, the formation of heterocysts at high frequency in NO_3^- plus tungsten medium seems to be due to a blockage of assimilation of NO_3^- owing to the formation of defective nitrate reductase enzyme. Thus, failing to utilize NO_3^-, the alga switches on its nitrogen-fixing machinery resulting in the formation of heterocysts. But, again, the tungsten present does not permit normal activity of nitrogenase and consequently the heterocyst frequency continues to increase. Formation of heterocysts without nitrogenase activity as indicated by acetylene reduction in nitrogen-free and nitrate-containing media following tungsten treatment suggests that: (1) active nitrogenase is not necessary for heterocyst production, or heterocyst formation is not always associated with nitrogenase activity; (2) nitrate per se does not inhibit heterocyst differentiation; rather, active nitrate reductase regulates heterocyst differentiation; and (3) nitrogenase and nitrate reductase enzymes (even defective) are not degraded rapidly as the restoration of their activity following molybdenum addition occurs very fast.

It may be concluded that by employing tungsten, one can manipulate the formation, activity, and properties of molybdoenzymes operative in cyanobacteria.

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